—Full Paper—

Gene Expression in Individual Bovine Somatic Cell Cloned Embryos at the 8-cell and Blastocyst Stages of Preimplantation Development

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Abstract. Aberrant gene expression in somatic cell nuclear-transferred (NT) embryos due to abnormal epigenetic modifications of the donor nucleus likely accounts for much of the observed diminished viability and developmental abnormalities. We compared the expression of 13 developmentally important genes in individual 8-cell and blastocyst stage NT embryos produced from adults female cumulus cells and adult male skin fibroblast cells with low and high incidences of neonatal abnormalities [1, 2]. In vitro-fertilized (IVF) embryos were used as control embryos. Among the genes tested, the relative abundance of Glut-1, IGF-1R, E-cad, and Cx43 transcripts varied significantly between the two types of NT embryos at the 8-cell stage. The relative abundance of manganese super oxide dismutase (MnSOD) and Stat3 transcripts was significantly higher in IVF embryos compared with both types of NT embryos. At the blastocyst stage, there was a significant difference in the relative expression of only one gene, Bcl-2, between the two types of NT embryos. Although the level of Glut-1 expression did not vary between the two types of NT blastocysts, its expression in both types of NT blastocysts was significantly lower than that in IVF blastocysts. The MnSOD expression level tended to be higher in NT blastocysts. The gene expression profile for any single gene, however, was highly variable among individual embryos and was independent of embryo morphology. The present study demonstrated that the expression profiles of the 13 genes examined in Day 9 NT blastocysts produced from two different types of donor cells with different incidences of neonatal abnormalities are largely indistinguishable.

Key words: Bovine, Embryo, Gene expression, Morphology, Somatic cell nuclear transfer (SCNT)

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Somatic cell nuclear transfer (SCNT) is generally successful, but its overall efficiency is very low despite nearly a decade of sustained effort to improve it since the first successful report in mammals [3, 4]. Cloned embryos die at various stages of development, including after birth, due to a variety of developmental defects. Most nuclear-transferred (NT) animals that do survive early neonatal life and their subsequent generations[5, 6], however, develop to apparently normal adults and have normal products [7]. Therefore, if methods can be devised for prior selection of NT embryos with the potential to develop into normal offspring, this technology would be of enormous benefit for animal production conservation practices, and human therapeutics.

Several lines of evidence suggest that epigenetic reprogramming during nuclear transfer, a process in which gene expression of a differentiated donor...
cell is reset to an early embryonic totipotent state, is often haphazard and stochastic, and this is believed to be responsible for the developmental defects of clones. The fact that these developmental defects are not passed on to their offspring reinforces the idea that the developmental abnormalities are caused by epigenetic defects [8–10]. Epigenetic markers confer stability in gene expression during mammalian development. Therefore, any changes or defects in epigenetic markers are likely to be reflected in the gene expression profile. Thus, gene expression analysis has become a useful tool for understanding the efficiency of nuclear reprogramming in SCNT embryos. Many recent reports suggest that a variety of gene expression patterns are abnormal in SCNT-derived neonatal tissues [10, 11], placentae [12] and preimplantation embryos [13–22]. Nevertheless, in all these studies except for a few reports [21, 22], NT embryos were compared with either their in vitro- or in vivo-derived counterparts. Studies comparing gene expression in NT embryos produced from different types of somatic cells [21, 23], however, are limited. Because no particular type of donor cell has been established to be superior in overcoming the problems associated with nuclear transfer and because a later study by Jang et al. [23] did not include control group embryos as a reference for normal embryos, the findings of Jang et al. [23] are not conclusive with regard to the differential expressions of genes among NT embryos from different types of donor cells. Furthermore, the potential of the donor cells used in their study to develop into live calves is not known. To date, the majority of studies concerning gene expression analysis of bovine NT embryos have involved pooled samples of blastocysts and/or have used semi-quantitative polymerase chain reaction (PCR) methods [13–18, 21, 23]. In view of the fact that the majority of NT blastocysts do not develop to term, pooling of NT samples might bias the results in favor of non-viable embryos.

Therefore, to bridge these gaps, in the present study we used a highly sensitive real-time PCR [21] to compare the gene expression profiles of a set of developmentally important genes in individual 8-cell and blastocyst stage NT embryos developed from adult female cumulus cell nuclear-transferred (CNT) oocytes, which have a low incidence of perinatal abnormalities, and from adult male skin fibroblast cell nuclear-transferred (FNT) oocytes, which have a high incidence of neonatal abnormalities [1, 2]. We thought that gene expression analysis at the 8-cell and blastocyst stages of preimplantation development might offer better insight into the reprogramming process. The set of genes chosen in this study have an important role in embryonic and fetal development [21]. The genes evaluated include genes involved in metabolism (Glut-1); pre- and post-natal growth (IGF-1R); pluripotency (Oct4); morphogenesis and biogenesis of the trophoderm (E-cad); lineage determination and cavitation (Stat3); gap junctions, cavitation, and cryosurvival (Cx43); heat (Hsp70.1) and oxidative (MnSOD) stress; DNA methylation (DNMT1); pro-apoptosis (Bax); anti-apoptosis (Bcl-2); implantation (FGF4) and growth arrest (CHOP-10). Furthermore, we show here that target gene expression profiles are highly variable among individual embryos and are independent of embryo morphology.

Materials and Methods

Preparation of donor cells

Donor cell lines that were previously established and frozen [1, 2], cumulus cells from an adult Japanese beef female and skin fibroblast cells from an adult Japanese beef male, were used in the present study. Although the pregnancy and calving rates after transfer of the two types of cloned blastocysts were not different, 6 of the 9 calves cloned from the cumulus cells survived to adulthood, and all 5 calves cloned from fibroblast cells died around parturition [1, 2]. Before nuclear transfer, frozen donor cells were removed from liquid nitrogen, thawed rapidly and cultured as described previously [21]. Donor cells from passages 6 through 12 were used in this experiment. Cumulus or fibroblast cells were induced to the G0/G1 stage by contact inhibition in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for 5 to 7 d [24].

Embryo production by nuclear transfer

In vitro-matured oocytes with a first polar body were treated with 0.5 µg/ml demecolcine (Wako, Osaka, Japan) for 30 to 60 min to induce a membrane protrusion containing condensed maternal chromosomes [25]. A cut was then made in the zona pellucida above the membrane protrusion using a sharp needle. The protrusion containing
the maternal chromosomes and a first polar body were gently squeezed out through the cut. A single cumulus or fibroblast cell was introduced into the perivitelline space of the enucleated oocyte and simultaneously electrically fused and activated by two direct current (150 V/mm) 25 µs pulses at a 0.1 s intervals in 0.28 M mannitol medium supplemented with 0.1 mM MgSO₄ and 0.05 mM CaCl₂ [26]. The fused oocytes were cultured in CR1-aa medium with 0.3% bovine serum albumin (fatty acid free) containing cycloheximide (10 µg/ml) for 5 h followed by cultured in cycloheximide-free medium until day 3 (day 1=day of nuclear transfer). On the third day, the embryos were moved into CR1-aa medium supplemented with 10% FBS and cultured until day 9 under the same atmosphere conditions.

**In vitro fertilization**

In vitro fertilization (IVF) was performed as described previously [27]. Oocytes (n=10–15) from an unknown breed were matured for 22 to 24 h and placed in 50 µl Brackett-Oliphant medium [28] after being washed twice in the same medium. Straws containing frozen semen from Japanese Black cattle were thawed in a water bath at 37 C. The spermatozoa were washed twice by centrifugation at 600 x g for 5 min in modified Brackett Oliphant medium containing caffeine (5.5 mM) and heparin (2.6 IU/ml), and the concentration was adjusted to 2 x 10⁶/ml. A 50 µl aliquot of semen suspension was added to each 50 µl droplet containing oocytes, and they were then co-cultured together for 6 h in a 5% CO₂ incubator at 39 C. After 6 h of co-incubation, cumulus cells were removed by repeated pipetting and cultured for 9 days (day 1=day of IVF) using the same culture protocol as described for the NT embryos.

**Donor cell and embryo collection and reverse transcription**

Morphologically normal 8-cell and blastocyst stage IVF, CNT and FNT embryos collected 44 to 48 h and 9 d after activation or insemination and donor cells were separately treated using a Cells-to-cDNA II kit (Ambion, Austin, TX, USA) according to a previous described procedure [21] immediately after taking photographs. Twelve to 16 embryos at each stage were analyzed in each group. Reverse transcription (RT) PCR was performed using the Moloney murine leukemia virus (MMLV) reverse transcriptase included in the Cells-to-cDNA™II kit under the following conditions. In a total reaction volume of 20 µl, there was 4.0 µM random hexamer primer, 1 µl reverse transcription buffer, 10 IU RNase inhibitor, 100 IU MMLV reverse transcriptase enzyme and 1 mM of each dNTP. Tubes were heated to 70 C for 3 min to denature the secondary RNA structure before adding the MMLV. To normalize the efficiency of the RT reaction, histone H2a mRNA levels were measured for each tube. The reaction mixture was then incubated at 42 C for 60 min, followed by 92 to 95 C for 10 min to inactivate the reverse transcriptase enzyme. Negative control reactions were performed in the absence of RNA or reverse transcriptase.

**Quantitative real-time PCR**

Quantification of all transcripts was performed by real-time quantitative PCR. PCR was performed using an ABI 7000 PRISM system (Applied Biosystems, Tokyo, Japan). The target transcript was evaluated using a dual-labeled probe designed to have a 5’ reporter dye (6-FAM) and a 3’ quenching dye (TAMRA). The sequences and Gene Bank accession numbers of the primer and probe set used for amplification of the target genes are presented in Table 1. The PCR reaction mixture (25 µl) contained 12.5 µl Taqman 2X PCR Master Mix (Roche, Branchburg, NJ, USA), 100 nM of each primer and probe, and 0.1 to 0.3 embryos in each tube. The thermal cycling conditions using the dual-labeled probe were 50 C for 2 min and 95 C for 10 min followed by 40 cycles of 95 C for 15 s and a combined annealing/extension stage of 60 C for 1 min. Histone H2a mRNA was assayed to correct for loading discrepancies. At least three sets of embryos were analyzed for each gene examined. All PCRs were conducted at least twice for every transcript of interest.

The comparative C₇ method was used for relative quantification of target gene expression levels (ABI Prism Sequence Detection System, Applied Biosystems). The quantification was normalized to the internal control histone H2a gene. Within the log-linear phase region of the amplification curve, each cycle doubled the amplified PCR product. The ΔC₇ value was determined by subtracting the histone H2a C₇ value for each sample from the tar-
get gene $C_T$ value of the sample. The calculation of $\Delta C_T$ involved using the highest sample $C_T$ value as an arbitrary constant to subtract from all other $C_T$ sample values. Fold-changes in the relative mRNA expression of the target were determined using the formula $2^{-\Delta\Delta C_T}$.

**Statistical analysis**

Relative quantification of target gene expression levels as fold-differences was based on at least three sets of samples. The mRNA expression data were analyzed using the Instat 2 software (Graphpad Software, San Diego, CA, USA). One-way repeated-measures analysis of variance followed by multiple pairwise comparisons using the Student-Newman-Keuls Multiple Comparisons Test was used for analysis of differences in mRNA expression assayed by quantitative real-time PCR. The embryo development data was analyzed using Chi-square test. A P value of less than 0.05 was considered to be significant.

**Table 1. Details of the primers and probes used for real-time RT PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers and probes</th>
<th>Sequence (5'–3')</th>
<th>Position on sequence</th>
<th>Accession number</th>
</tr>
</thead>
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<tr>
<td>E-cad</td>
<td>Forward</td>
<td>GGACTTGTGACCTGAGCCAGTTTACAT</td>
<td>84–108</td>
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<td></td>
<td>Reverse</td>
<td>TGGTGCACGTCATGTC</td>
<td>157–140</td>
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<td></td>
<td>Probe</td>
<td>CAGGCCAGGAGCATCAGCC</td>
<td>130–111</td>
<td></td>
</tr>
<tr>
<td>Stat3</td>
<td>Forward</td>
<td>CAGGAAATGATGAACTGATACTGGAAGA</td>
<td>1195–1218</td>
<td>AJ620655.1</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>CTCTGCTCTTCCTCCGAGGTCACA</td>
<td>1278–1256</td>
<td></td>
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<tr>
<td></td>
<td>Probe</td>
<td>TCAAGAACGCACTACCTGAGCCA</td>
<td>1220–1245</td>
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<td>IGF-1R</td>
<td>Forward</td>
<td>GCAGATGGCAGATGCATACCT</td>
<td>1177–1196</td>
<td>XS4980</td>
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<td>Reverse</td>
<td>ATCCTGCGGCCACCTAGCA</td>
<td>1257–1240</td>
<td></td>
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<tr>
<td></td>
<td>Probe</td>
<td>CCAAAAGATTTTGTCCAACAGACCTGCCTG</td>
<td>1202–1231</td>
<td></td>
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<td>Glut-1</td>
<td>Forward</td>
<td>GAGTACCTTCCCCACACGTAAC</td>
<td>534–552</td>
<td>M60448</td>
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<td></td>
<td>Reverse</td>
<td>TGCACCTGGGCTGACCTGGTTT</td>
<td>562–587</td>
<td></td>
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<tr>
<td>MnSOD</td>
<td>Forward</td>
<td>CTCTGCAAGCTGCTGGTGA</td>
<td>275–293</td>
<td>L22092</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>GTCAAGTTTGAATGCTTCCA</td>
<td>399–380</td>
<td></td>
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<tr>
<td></td>
<td>Probe</td>
<td>CTTCGGTTTTCACCTACCGTTAG</td>
<td>370–346</td>
<td></td>
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<tr>
<td>CHOP-10</td>
<td>Forward</td>
<td>GGCCACTTTCGACTCCCTCC</td>
<td>701–720</td>
<td>NM001078163.1</td>
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<td>Reverse</td>
<td>CCCTTGCTGAATGCTAGGTT</td>
<td>786–768</td>
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<td>Probe</td>
<td>ACCACGTCACCAGTGCAACGAC</td>
<td>735–759</td>
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<td>Bcl-2</td>
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<td>Probe</td>
<td>CGGGCCTGTTGAGTTTCTCGAG</td>
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<td>Bax</td>
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<td>GCCGATGGAGATGAGATTG</td>
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<td>Reverse</td>
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<td>321–301</td>
<td></td>
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<tr>
<td></td>
<td>Probe</td>
<td>TGTCACGACGGTCCTTTCCAC</td>
<td>253–229</td>
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<tr>
<td>FGF-4</td>
<td>Forward</td>
<td>TACGGCTGCTTCCCTTCTCAC</td>
<td>436–455</td>
<td>U15969</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>TTGGCTTCGGTCGTTCTGG</td>
<td>563–546</td>
<td></td>
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<tr>
<td></td>
<td>Probe</td>
<td>CCTCTCCCAAGACTCAAACGGCTTACGAG</td>
<td>483–510</td>
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<tr>
<td>DNMT1</td>
<td>Forward</td>
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<td>3794–3814</td>
<td>AY173048</td>
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<td></td>
<td>Reverse</td>
<td>GCCAAAGGTGCGACTGGTACC</td>
<td>3882–3865</td>
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<td></td>
<td>Probe</td>
<td>TCCATGGTCTCTGAAGCTGAGCTG</td>
<td>3820–3843</td>
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<td>Hsp70.1</td>
<td>Forward</td>
<td>ATGACCCGCTGTGATCAAG</td>
<td>1228–1246</td>
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<td>Reverse</td>
<td>CCTCTGGACAGTTCCGACCAA</td>
<td>1333–1313</td>
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<td></td>
<td>Probe</td>
<td>CAATACTCCATCCCCCAGAAGCA</td>
<td>1248–1271</td>
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<tr>
<td>Cx43</td>
<td>Forward</td>
<td>GACAAATCCTCTTCTTCAATCCTC</td>
<td>209–232</td>
<td>NM174068</td>
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<td>CCTTGGCACTGTAGAAGAACAC</td>
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<td>Probe</td>
<td>TGGTCCCAACCTCTGTACCTGCGCTAT</td>
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<td>Oct-4</td>
<td>Forward</td>
<td>CCTCGAGCAAAAATTTGAGCAGCA</td>
<td>1294–1314</td>
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<td>Reverse</td>
<td>CAGTTGGAGATGACTGCTCCTTT</td>
<td>1517–1495</td>
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<td></td>
<td>Probe</td>
<td>TGCAAAACACACTGGAGACCAGT</td>
<td>1469–1448</td>
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</table>
Table 2. In vitro development of NT and IVF oocytes

<table>
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<tr>
<th>Type</th>
<th>No. of oocytes cultured</th>
<th>2-cell</th>
<th>4-cell</th>
<th>8-cell</th>
<th>Blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNT</td>
<td>1160</td>
<td>908 (78)</td>
<td>673 (58)</td>
<td>410 (35)</td>
<td>262 (23)</td>
</tr>
<tr>
<td>FNT</td>
<td>1173</td>
<td>922 (79)</td>
<td>723 (62)</td>
<td>468 (40)</td>
<td>253 (22)</td>
</tr>
<tr>
<td>IVF</td>
<td>411</td>
<td>298 (73)</td>
<td>215 (52)</td>
<td>166 (40)</td>
<td>87 (21)</td>
</tr>
</tbody>
</table>

Results

Table 2 shows the development of the IVF and NT oocytes with the two different types of somatic cells. The potential to develop to the 2-cell, 4-cell, 8-cell and blastocyst stages was not different among the groups.

The genes evaluated in the present study were differentially expressed in the cumulus and fibroblast cells (Fig. 1). Transcripts for Oct-4 and FGF4 were not observed in either type of donor cell. All of the remaining genes were transcriptionally active in both types of donor cell. Although the expressions of the active genes were more or less similar in cumulus cells, they varied substantially in fibroblast cells. There was a several-fold difference in the gene expression levels between the two donor cell types, except for the genes encoding Cx43, Glut-1 and IGF-1R.

Of the 13 genes evaluated at the 8-cell stage (Figs. 2 and 3), the relative abundance of transcripts for Cx43, Glut-1, IGF-1R and E-cad was significantly different between the two types of NT embryos. The expression levels of Glut-1 and IGF-1R were significantly higher in the FNT embryos compared with the CNT embryos and IVF-derived embryos. However, there was no difference in the expression of these two genes between the CNT and IVF-derived embryos. The CNT embryos had different expression levels of E-cad and Cx43 compared with the FNT- and IVF-derived embryos. Although E-cad expression was significantly higher in the CNT embryos, Cx43 expression was significantly lower.

The relative abundance of Stat3 and MnSOD transcripts was significantly higher in the IVF embryos compared with either type of NT embryo. There were no differences in the expression of the mRNA transcripts of the remaining genes (Oct4, CHOP-10, DNMT1, Hsp70.1, Bax and Bcl-2) between the two types of NT embryos or between either type of NT embryo and IVF embryos. FGF-4 gene transcripts were not detected in the NT or IVF-derived 8-cell embryos.

Among the 13 genes evaluated at the blastocyst stage (Figs. 2 and 4), there was a significant difference in the relative expression of only one gene, Bcl-2, between the two types of NT embryos (Fig. 4). Bcl-2 expression was significantly lower in the CNT-derived blastocysts compared with the FNT-derived blastocysts. However, the expression level in the FNT blastocysts was similar to that in the IVF embryos.
Fig. 2. Relative transcript abundance analyzed by RT-PCR at the 8-cell (8C) and blastocyst (BL) stages in individual embryos derived from \textit{in vitro} fertilization (IVF) cumulus (CNT) and fibroblast (FNT) nuclear transfer.
Fig. 2. Continued
blastocysts. Although the Glut-1 expression level did not differ between the two types of NT blastocysts, the expression in both types of NT blastocyst was significantly lower than that in the IVF blastocysts. Furthermore, although there was no difference in the level of MnSOD expression between the two types of NT blastocysts, the CNT-derived blastocysts had significantly higher expression levels than the IVF blastocysts. The majority of IVF-derived (8/12, 67%) blastocysts expressed FGF-4, but only 23% (3/13) of the CNT-derived blastocysts and 27% (4/15) of the FNT-derived blastocysts expressed FGF-4.

Comparison of blastocyst morphology with their
Fig. 3. Average relative transcript expression levels of *in vitro* fertilized (IVF) and cumulus (CNT) and fibroblast (FNT) nuclear-transferred 8-cell embryos. Bars with different superscripts within a panel differ significantly (P<0.05). The standard deviation is indicated by error bars.
Fig. 4. Average relative transcript expression levels of *in vitro*-fertilized (IVF) and cumulus (CNT) and fibroblast (FNT) nuclear-transferred blastocysts. Bars with different superscripts within a panel differ significantly (*P*<0.05). The standard deviation is indicated by error bars.
respective gene expression profiles indicated that blastocyst morphology did not correlate with the changes in the target gene expression levels. The gene expression levels of some blastocysts with similar morphology varied substantially for all genes examined. As an example, the variations in MnSOD expression levels between embryos with similar morphology are shown in Fig. 5.

**Discussion**

The genes evaluated in the present study have a critical role in early embryonic development [15, 29–31], and the majority of the genes are expressed maternally as well as embryonically [32]. We thought that gene expression analysis at the 8-cell and day 9 blastocyst stages for two different types of NT embryos might offer better insight into the reprogramming process and gene expression patterns of preimplantation NT embryos. It is possible that gene expression analysis at the 8-cell and day 9 blastocyst stages in the present study might correspond to gene expression analysis before and after the maternal-to-zygotic transition (MZT), respectively. A major surge in the synthesis of new RNA corresponding to the MZT in bovine embryos occurs during the second half of the third and fourth cell cycle. A distinct surge in new RNA synthesis occurs at the 8-cell stage, 66 h after insemination [33]. Furthermore, the MZT in the bovine is thought to be controlled temporally rather than by the embryonic stage [34]. Recently, Camargo et al. [35] used 8-cell stage embryos col-

![Figure 5](image)

Fig. 5. Variations in the MnSOD expression level between embryos with similar morphology. IVF, CNT and FNT represent *in vitro*-fertilized and cumulus and fibroblast nuclear-transferred blastocysts.
lected 70 to 74 h post-activation for gene expression analysis of bovine NT embryos at MZT. Therefore, in the present study, as an attempt to examine the gene expression pattern before the MZT, we collected 8-cell embryos 44 to 48 h after activation or insemination. As discussed below, the results of the present study support our hypothesis. To the best of our knowledge, there are no reports comparing gene expression in NT bovine embryos before and after the MZT.

While the exact reasons for the differential expression of these genes between donor cell types is unknown, differences in epigenetic markers, such as DNA methylation, histone tail modifications, and binding of non-histone proteins to chromatin, between the different types of cells [36–39] could be responsible for the differential expression of these genes. Subsequent to nuclear transfer, however, all the genes that were differentially expressed with a several-fold difference between the two types of donor cells, except for E-cad, had similar levels of expression in the 8-cell NT embryos produced from these donor cells. Furthermore, the relative levels of expression of these genes in both types of NT embryos were comparable to those in the IVF-derived 8-cell embryos. This supports the notion that oocytes have a unique potential to reprogram the gene expression patterns of differentiated donor cells with a different epigenetic make-up to that of the embryonic state, thereby supporting early embryonic development, and in some cases, development to term [40].

The genes encoding Cx43, Glut-1, IGF-1R and E-cad were differentially expressed between the two types of NT 8-cell embryos. Although persistent execution of the donor cell program and/or possible differences in the physiology and metabolism of the NT embryos produced from different types of donor cells [41] might be responsible for the differential expression of these four genes, it is also possible that the differences in the transcriptional status of the donor genome are a consequence of its initial reprogramming/remodeling. Mouse oocytes activated before the MZT are transcriptionally permissive, and the ability of a foreign nucleus to induce reprogramming in the first cell cycle depends on the internal readiness of the cell to perform RNA synthesis rather than on the cytoplasmic environment [42]. The differences might also be due to enhanced or delayed degradation of the maternal pool of transcripts for Cx43, Glut-1, IGF-1R and E-cad, as these four genes are expressed throughout embryonic development in in vitro-produced bovine embryos [32]. The differences in expression of these four genes between the two types of NT embryos decreased at the blastocyst stage, and the expression levels did not differ from those of their in-vitro produced counterparts, suggesting that the MZT, which should have occurred a little later, at the late 8-cell to 16-cell stage, could have restored the normal embryonic pattern of expression of these genes. Therefore, it is possible that the differential expression levels of these four genes at such an early stage do not have serious implications for the subsequent development of NT-derived embryos produced from these cells because no differences were observed either in their potential to develop to the blastocyst stage [present study], blastocyst cell numbers [21], cryosurvival potential (unpublished observation) or ability to develop to term [1, 2].

Of the 13 genes evaluated at the blastocyst stage, only one gene encoding Bcl-2, an anti-apoptosis gene, was differentially expressed between the two types of NT-derived blastocysts. Compared with the FNT embryos, Bcl-2 expression was downregulated in the CNT embryos, which is consistent with previous reports [21, 23]. Although not tested in the present study, we speculate that the higher expression of the X-linked inhibitor of apoptotic protein (XIAP), an important modulator of caspase activity [43] in female bovine blastocysts compared with male blastocysts [44], might be responsible for or might have compensated for the low expression of Bcl-2 in the female CNT-derived blastocysts compared with the male FNT-derived blastocysts. In support of our hypothesis, a separate experiment using NT embryos produced from the same line of cumulus cells as those used in the present study and a different line of cumulus cells showed that higher XIAP expression in all CNT-derived embryos (data not shown). Although it is highly speculative, the differential expression of Bcl-2 between the two types of NT embryos seems to be a consequence of sex differences rather than differences in the epigenetic makeup of the donor cells used. There were no differences in the relative expression levels of the remaining genes between the two types of NT-derived embryos. Our results are in contrast to those of previous reports [21–23] in which there was differential expression of genes between NT embryos produced from different
types of donor cells. In all of these previous reports, however, pooled samples were used for gene expression analysis, whereas individual embryos were used in the present study. We suspect that pooling of NT samples skews the results in favor of non-viable embryos, as most NT embryos fail to develop to term.

FGF-4 is essential for trophoblast growth and is directly or indirectly responsible for survival of the inner cell mass (ICM) of blastocysts [45]. In mice, null mutant embryos for FGF-4 or its receptor FGFr2 die around the time of implantation as a consequence of ICM death or failure of placental growth [46–48]. Although FGF-4 transcripts were not detected in any type of 8-cell embryos tested, 67% of the IVF-derived blastocysts (8/12), 23% of the CNT blastocysts (3/13) and 27% of the FNT (4/15) blastocysts contained transcripts for FGF-4, which is consistent with previous reports on bovine NT embryos [14]. A previous report [49] suggested that nearly 60% of IVF and 30% of NT-derived blastocysts have a normal ratio of ICM to total cells; interestingly, these figures are consistent with the percentage of blastocysts expressing FGF4 observed in the present study. Although blastocyst morphology did not correlate with the FGF4 expression level in all groups, it remains to be elucidated whether the absence of FGF4 expression has any influence on blastocyst cell number in the bovine. It has been suggested that FGF4 is essential for cellular proliferation, ICM survival [46], initial growth of the placental lineage [50] and specification of the trophoderm and primitive endoderm [51] in mouse development. It is largely unknown whether FGF4 has a similar role in the bovine. Considering that 40 to 60% of IVF and 10 to 20% of NT embryos from bovine develop to term, the extensive loss of NT embryos around the time of implantation in bovine and the important role that FGF4 has in trophoderm specification and initial growth of the placental lineage in mice, it is possible that the absence or reduction of FGF4 in the majority of bovine NT blastocysts affects embryonic mortality around the time of implantation. Therefore, it would be interesting to elucidate the role of FGF signaling in bovine embryonic development and placental growth to determine whether FGF4 can be used as a marker for selecting viable embryos in vitro.

The higher expressions of Stat3, which is involved in cell lineage determination, and MnSOD, an oxidative stress response gene, in the IVF-derived 8-cell stage embryos compared with either type of NT embryo might be due to early activation of these genes during IVF at the minor MZT [33, 52], which occurs at the 2 to 4-cell stage in bovine. Stat3 null mutant embryos develop into blastocysts, but degenerate immediately after implantation [53]. Stat3 has been suggested to be involved in determination of the animal pole of the oocyte and in establishment of the ICM and trophoderm (TE) cell lineages in preimplantation stage human and mouse embryos [54]. Although it is speculative, the low abundance of Stat3 in NT-derived 8-cell embryos might affect polarization of this protein in early cleavage-stage embryos and may affect the subsequent allocation of ICM and TE cells, which is aberrant in bovine SCNT blastocysts [49].

In contrast to the expression pattern at the 8-cell stage, MnSOD expression was higher in the NT blastocysts, particularly in the CNT blastocysts, compared with the IVF blastocysts. Although not significant, the expression level was relatively higher in the FNT-derived blastocysts compared with the IVF-derived counterparts. Because MnSOD is implicated in promoting cellular differentiation [55], the higher expression in the NT blastocysts could be due to inadequate reprogramming or de-differentiation of the donor genome in the NT embryos. The aberrant expression of this gene both at the 8-cell and blastocyst stages in NT embryos suggests that NT embryos fail to establish an appropriate embryonic MnSOD expression pattern. The higher expression of MnSOD in in vivo embryos, embryos cultured in vivo in the sheep oviduct and in vitro embryos surviving vitrification suggests that developmentally viable embryos contain higher MnSOD expression [56, 57]. The present study suggests that this might not hold true for NT-derived bovine embryos, because of their poor in vivo developmental potential.

There was a significant difference in the expression of Glut-1 between the NT and IVF-derived blastocysts. Previous reports in the mouse suggest that, compared with in vivo blastocysts, Glut-1 expression decreases by 50% in in vitro-derived blastocysts [58]. Furthermore, freezing and thawing abolishes Glut-1 expression, leading to poor development of frozen-thawed embryos [59]. These reports clearly suggest that viable embryos have higher Glut-1 expression. The decrease in the
relative expression of Glut-1 in both types of NT blastocysts supports the notion that NT embryos are inferior to IVF embryos in their ability to develop to term. Decreased glucose transporter expression triggers BAX-dependent apoptosis in mouse blastocysts [60]. Therefore, it is possible that the low level of Glut-1 expression observed in the NT embryos is causally related to the high incidence of apoptosis in bovine NT-derived blastocysts [61].

Blastocyst morphology is quite often used as a criterion to select viable embryos produced in vitro for embryo transfer in assisted reproductive technologies. Embryo transfer of the selected IVF embryos allows for an average of 30% of the transferred embryos to develop to term in the bovine [62]. Even in IVF embryos, however, development to term is highly variable, in the range of 12 to 50% [62]. Comparisons of morphology with the gene expression profile suggest that differences in the gene expression profile among morphologically similar embryos may contribute to the variations observed in their potential to develop to term. It is largely unknown, however, what contributes to the variations in the target gene expression among morphologically similar embryos. Therefore, it is possible that the gene expression level(s) for all or some of the genes we examined do not always correlate with embryo morphology.

In conclusion, with the exception of Bcl-2, the expression patterns of the studied genes were indistinguishable between the two types of NT-derived blastocysts. We hypothesize that if the observed differences in potential to develop into live calves among the NT-derived embryos produced from different donor cells was due to differences in their gene expression patterns during preimplantation development, these differences might be subtle [35, 63, 64] at the blastocyst stage or might develop during later stages of development in vivo [65].

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